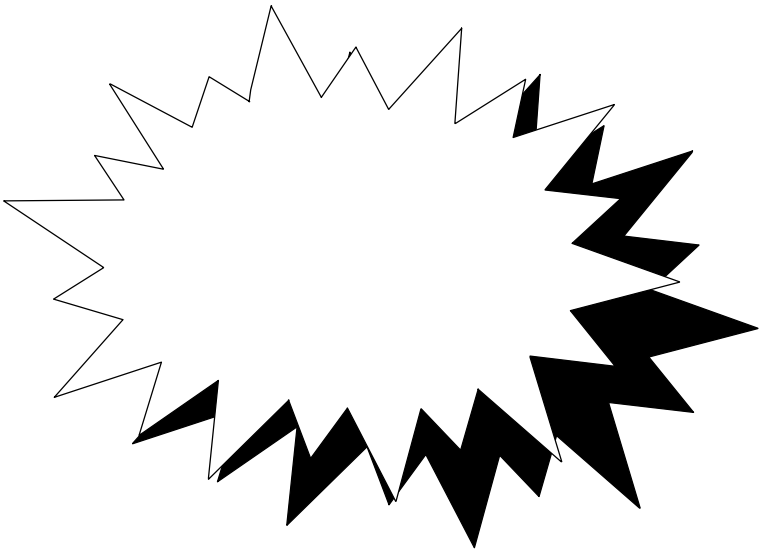

Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-016

Procedure Title: Determination of Starch in Biomass Samples by Chemical Solubilization and Enzymatic Digestion

Author: Tina Ehrman

Date: 5/29/96

ISSUE DATE: 9/25/96 **SUPERSEDES:** n/a

DISCLAIMER

These Standard Biomass Analytical Methods (“Methods”) are provided by the National Renewable Energy Laboratory (“NREL”), which is operated by the Midwest Research Institute (“MRI”) for the Department Of Energy.

Access to and use of these Methods shall impose the following obligations on the user. The user is granted the right, without any fee or cost, to use, copy, modify, alter, enhance and distribute these Methods for any purpose whatsoever, except commercial sales, provided that this entire notice appears in all copies of the Methods. Further, the user agrees to credit NREL/MRI in any publications that result from the use of these Methods. The names NREL/MRI, however, may not be used in any advertising or publicity to endorse or promote any products or commercial entity unless specific written permission is obtained from NREL/MRI. The user also understands that NREL/MRI is not obligated to provide the user with any support, consulting, training or assistance of any kind with regard to the use of these Methods or to provide the user with any updates, revisions or new versions.

THESE METHODS ARE PROVIDED BY NREL/MRI "AS IS" AND ANY EXPRESS OR IMPLIED WARRANTIES, INCLUDING BUT NOT LIMITED TO, THE IMPLIED WARRANTIES OF MERCHANTABILITY AND FITNESS FOR A PARTICULAR PURPOSE ARE DISCLAIMED. IN NO EVENT SHALL NREL/MRI BE LIABLE FOR ANY SPECIAL, INDIRECT OR CONSEQUENTIAL DAMAGES OR ANY DAMAGES WHATSOEVER, INCLUDING BUT NOT LIMITED TO CLAIMS ASSOCIATED WITH THE LOSS OF DATA OR PROFITS, WHICH MAY RESULT FROM AN ACTION IN CONTRACT, NEGLIGENCE OR OTHER TORTIOUS CLAIM THAT ARISES OUT OF OR IN CONNECTION WITH THE ACCESS, USE OR PERFORMANCE OF THESE METHODS.

Determination of Starch in Biomass Samples by Chemical Solubilization and Enzymatic Digestion

Laboratory Analytical Procedure #016

1. Introduction

1.1 Starch is a high molecular weight polymer consisting of glucose units linked by α-glucosidic bonds. Starch consists of two glucose polymers, amylose and amylopectin. Amylose is a linear polymer of glucose linked through α-D-1,4-glucosidic bonds while amylopectin is a branched polymer consisting of α-D-1,4-glucosidic bonds with a small number of α-D-1,6-glucosidic linkages present as interchain branch points. The relative proportions of these polymers varies with the source, but typically contains 15 to 25% amylose and 75 to 85% amylopectin. Upon hydrolysis, starch is broken down to a spectrum of higher and lower molecular weight oligosaccharides. Complete enzymatic hydrolysis yields D-glucose, which can be analyzed using an immobilized enzyme (glucose oxidase) technique.

$$\text{starch} + H_2O \xrightarrow{\text{amyloglucosidase}} \beta\text{-D-glucose (dextrose)}$$

$$\text{glucose} + O_2 \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{D-glucono-}\delta\text{-lactone}$$

1.2 Amyloglucosidase (glucoamylase) is an exoglucosidase which catalyzes the hydrolysis of both the α-D-1,6-glucosidic branch points and the predominating α-D-1,4-glucosidic linkages of starch. Amyloglucosidase removes glucose units successively from the nonreducing ends of starch chains and dextrins. The rate of hydrolysis of the α-D-1,6-glucosidic branch points is much slower than the rate for the α-D-1,4-glucosidic linkages.

2. Scope

2.1 This method covers the determination of starch in biomass samples. Sample material suitable for this procedure include hard and soft woods, herbaceous materials, agricultural residues, waste-paper, washed acid- and alkaline-pretreated biomass, and the solid fraction of fermentation residues. All results are reported relative to the 105EC oven-dried weight of the sample. In the case of extracted materials, the results may also be reported on an extractives-free basis.

2.2 This procedure is suitable for air-dried, lyophilized, and extracted biomass samples, as well as for samples that have been oven dried at a temperature of 45°C or less. The assay results will be biased slightly low for samples dried at 105°C. If sample availability is limited, it may be necessary to run this analysis on a 105°C dried sample but the results must be flagged as being biased low.

2.3 The assay is also suitable for wet samples if the particle size is known to be small and if the moisture content of the sample can be estimated accurately enough to predict the amount of sample needed to give 0.5 g of solids.

2.4 All analyses shall be preformed according to the guidelines established in the Ethanol Project Quality Assurance Plan (QAP).

3. References

3.1 NREL Ethanol Project Laboratory Analytical Procedure #001, "Standard Method for the Determination of Total Solids in Biomass".

3.2 Solvay Enzymes. 1996. *Fungal glucoamylase for Starch Hydrolysis.* Diazyme L-200 Technical Notes.

3.3 YSI Incorporated. 1994. *Determination of Cook in Extruded Cereal Products.* Application Note #319.

4. Significance and Use

4.1 The percent starch content is used in conjunction with other assays to determine the total composition of biomass samples.

5. Interferences

5.1 Interference by free glucose and cellobiose present in samples is not a problem because both glucose and cellobiose are destroyed during the NaOH solubilization step.

6. Apparatus

6.1 Analytical balance, accurate to 0.1 mg.

6.2 YSI 2700 Select Biochemistry Analyzer - equipped with a YSI 2365 dextrose membrane and YSI 2357 buffer and calibrated with YSI 2776 2.5 g/L calibrator solution.

7. Reagents and Materials

7.1 Glucose calibration verification standards, such as YSI 2.0 and 9.0 mg/mL glucose standards.

7.2 Amyloglucosidase (suggested source, Sigma A-3042).

7.3 Amylopectin (suggested source, Sigma A-7780).

7.4 Methanol, ACS reagent grade.

7.5 Hot plate or a water bath set at $90^{o} \pm 2^{o}$C.

7.6 Graduate cylinders of appropriate sizes.

7.7 100 mL, 500 mL, and 1000 mL volumetric flasks, class A.

7.8 5 and 10 mL pipets or adjustable pipettor.

7.9 Timer.

7.10 Water bath set at 40^{o} " 1^{o}C.

7.11 Serum bottles or Erlenmeyer flasks, 125 mL.

7.12. Prepared reagents:

7.12.1 2N NaOH - weigh 40 grams of sodium hydroxide pellets into a 500 mL volumetric flask. Add 300 mL of reagent grade water and mix. Cool, dilute to volume and mix.

7.12.2 2N HCl - measure 82.4 mL of concentrated hydrochloric acid and transfer to a 500 mL volumetric flask. Let cool, dilute to volume with reagent grade water and mix.

7.12.3 Acetate buffer (pH 4.2) - weigh 9.1 grams of sodium acetate into 500 mL volumetric flask. Add about 300 mL of reagent grade water and mix until all the solid is dissolved. Add 22.3 mL (23.4 grams) of glacial acetic acid. Dilute to volume with water and mix.

7.12.4 Amyloglucosidase working solution - prepare a fresh working solution of the enzyme such that it contains 60 units of activity per milliliter. If using the Sigma A-3042 amyloglucosidase, dilute the solution one hundred-fold into cold reagent grade water. Prepare daily and store in the refrigerator.

7.12.5 25% TCA - dissolve 50.0 grams of trichloracetic acid in 200 mL reagent grade water.

7.12.6 Phosphate buffer - dissolve 40 g NaH_2PO_4 and 10 g Na_2HPO_4 in reagent grade water, bring to volume in a 1000 mL volumetric flask.

8. ES&H Considerations and Hazards

8.1 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

8.2 Trichloroacetic acid is a hazardous chemical; appropriate precautions must be taken.

9. Procedure

9.1 The sample must not contain particles larger than 0.25 mm in diameter. If milling is required to reduce the particle size of the test specimen, a laboratory mill equipped with a 40 mesh, or smaller, screen should be used. If the sample size is too small to allow the use of a laboratory mill, a coffee grinder may be used instead.

Note: If pretreated samples are to be analyzed, the sample must be thoroughly washed to remove any residual acid or alkali prior to drying.

9.2 Duplicate portions of each sample must be weighed out for a total solids determination (following LAP-001) at the same time as the portions for the starch determination. If this is done later, it can introduce an error in the calculation because ground biomass can rapidly gain or lose moisture when exposed to the atmosphere. Record the average total solids value as T_{final}.

9.3 Weigh out approximately 0.50 g of sample to the nearest 0.0001 g and transfer to a 125 mL serum bottle or Erlenmeyer flask. Record as W_{sample}, the initial sample weight. Each sample must be run in duplicate, at minimum.

9.4 A standard reference material, amylopectin, is run in parallel with each batch of samples and its calculated recovery used to correct the sample results for losses due to the procedure. Weigh 0.5 g portions of amylopectin to the nearest 0.0001 g and transfer to serum bottles or Erlenmeyer flasks. Record the weight as $W_{standard}$, the initial standard reference material weight. The standard must be run in duplicate, at minimum. As with the samples, the total solids content, T_{final}, of the standard reference material must also be determined.

9.5 Add 25 mL of reagent grade water to each bottle. Swirl to ensure the sample is wetted and evenly dispersed.

> Note: A few drops of methanol may be used to prewet the sample which will aid in its dispersion once the water is added.

9.6 Add 10 mL 2N NaOH to the solution in each bottle. Place bottles on a heating unit or in a water bath preheated to 90°C. Heat for 20 minutes, swirling periodically to wet any sample that may be clinging to the side of the bottle. A glass stirring rod may be needed to break up clumps of material.

9.7 Add 10 mL 2N HCl to each bottle and swirl to mix. Cool the bottles to below 50°C.

9.8 Add 10 mL of acetate buffer to each bottle and swirl to mix.

9.9 Add 5.0 mL amyloglucosidase working solution to each bottle. Mix well and place the bottles in a 40°C water bath for 60 minutes.

9.10 After 60 minutes incubation, remove the bottles from the water bath. Immediately add 5 mL of 25% TCA to each bottle to stop hydrolysis.

9.11 Cool to room temperature and transfer each hydrolyzate to a 100 mL volumetric flask. Rinse out the bottle with small volumes of phosphate buffer and transfer all the rinses to the volumetric flask. Bring to volume with phosphate buffer and mix well.

9.12 Since the enzyme solution may contain free glucose, an enzyme blank must be run in parallel with the samples. Dilute duplicate 5.0 mL portions of the amyloglucosidase working solution to 100 mL with reagent grade water in a volumetric flask. These enzyme blanks will be analyzed in the same manner as the sample, with the averaged results used to correct the glucose contents of the samples.

9.13 The sample itself may contain free glucose, which normally would be analyzed as starch. However in this procedure the glucose, and also cellobiose, is destroyed in the NaOH solubilization step. Therefore no correction for free glucose is needed when calculating the total starch content of a sample.

9.14 Set up and calibrate the YSI as described in the manufacturer's manual using the dextrose membrane, YSI 2357 system buffer, and YSI 2776 2.5 g/L calibrator solution. Program the instrument to autocalibrate every fourth sample or every fifteen minutes, set the sample size to 25 µL, and use the following probe parameters:

Chemistry - dextrose
Units - g/L
Calibrator - 2.50 g/L
End point - 30 seconds
Cal station # - 1

9.15 Verify the calibration of the YSI using the glucose calibration verification standards before starting the run. Reverify the calibration periodically during the analysis and at the end of the run.

9.16 Measure the glucose levels in the enzyme blanks and in all the samples. The validated linear range of the instrument is 0 - 9.0 g/L dextrose. If the value reported exceeds the validated range, the hydrolyzate must be diluted appropriately and rerun.

10. Calculations

10.1 Calculate the amount of starch recovered from each analysis of the amylopectin standard reference material as follows, on a 105°C dry weight basis, and then average the recoveries:

$$\%\ Standard\ recovered = \frac{(YSI_{standard}, g/L - YSI_{enzyme\ blank}, g/L)\ x\ total\ volume, L}{standard\ weight, g, W_{standard}\ x\ \frac{\%\ total\ solids, T_{final}}{100}}\ x\ 0.9\ x\ 100\%$$

Note: Amylopectin recoveries of 93 to 95% have routinely been achieved with this protocol. Recoveries less than 90% indicate the data generated for the batch of samples should be rejected and the analysis repeated.

10.2 Calculate the amount of starch present in each sample, on a 105°C dry weight basis:

$$\%\ Starch = \frac{(YSI_{sample}, g/L - YSIsubenzyme\ blank, g/L)\ x\ total\ volume, L}{sample\ weight, g, W_{sample}\ x\ \frac{\%\ total\ solids, T_{final}}{100}}\ x\ 0.9\ x\ 100\%$$

Note: The factor 0.9 converts grams of glucose to grams of the anhydrosugar (starch, in this case). The factor can be calculated by dividing the molecular weight of glucose less one molecule of water (180 - 18) by the molecular weight of glucose.

10.3 The calculated percent starch in each sample can be corrected for assay losses using the percent recovery of the standard reference material, amylopectin, as follows:

$$\%\ Starch, corrected = \frac{\%\ starch}{average\ \%\ standard\ recovered}\ x\ 100\%$$

11. Report

11.1 Report the percent starch present in the sample, to two decimal places, on a 105°C dry weight basis.

11.2 For replicate analyses of the same sample, report the average, standard deviation, and relative percent difference (RPD).

12. Precision and Bias

12.1 Data obtained by replicate testing of a corn stover in one laboratory gave a standard deviation of 0.28% and a CV of 1.31%. Data obtained by replicate testing of a amylopectin reference material in three laboratories gave a standard deviation of 0.58% and a CV of 4.61%.

12.2 This procedure has been validated for materials which have been air-dried, lyophilized, or oven dried at a temperature of 45°C or less. The assay results will be biased slightly low for samples dried at 105°C. If sample availability is limited, it may be necessary to run this analysis on a 105°C dried sample but the results must be flagged.

13. Quality Control

13.1 *Reported significant figures:* Report the percentage of starch present in the sample to two decimal places, on a 105°C dry weight basis, or extractives-free basis. Cite the basis used in the calculation.

13.2 *Replicates:* At minimum, all samples and the method verification standard are to be analyzed in duplicate.

13.3 *Blank:* As described in the "Procedure" section, enzyme blanks are prepared in duplicate and analyzed in the same manner as the samples.

13.4 *Relative percent difference criteria:* The RPD must be less than 8.0%. If the RPD is too large, the sample must rerun.

13.5 *Method verification standard:* In this procedure, amylopectin is used as both a method verification standard and as a means for estimating and correcting for assay losses. At minimum, the amylopectin must be run in duplicate with every batch of samples. If the recovery of amylopectin is less than 90%, the data generated for the whole batch of samples must be rejected and the analysis repeated.

13.6 *Calibration verification standard:* Calibration verification standards may be purchased from YSI or independently prepared. The CVSs are analyzed periodically through the YSI run as described in the "Procedures" section.

13.7 *Sample size:* A minimum of 3.6 grams of sample (on a dry weight basis) are required for duplicate analyses, which includes both the starch and the total solids assays. If there is insufficient sample, the result will be flagged and the lack of precision noted.

13.8 *Sample storage:* Dried samples shall be stored in an airtight container at room temperature. Samples with moisture contents greater than 10% shall be stored in an airtight container and refrigerated for not longer than one week. If longer periods of storage are required, these samples must be stored frozen.

13.9 *Standard storage:* YSI standards should be stored refrigerated and should not exceed the manufacturer's stated expiration data.

13.10 *Standard preparation:* Purchase the YSI 2776 2.5 g/L calibrator solution from YSI.

13.11 *Definition of a batch:* Any number of samples which are analyzed and recorded together. The maximum size of a batch would be limited by the equipment constraints.

13.12 *Control charts:* The result of each replicate analysis of the method verification standard (amylopectin) is recorded along with the average, RPD, and a laboratory book/page reference. The average value obtained for each analysis of the method verification standards is to be control charted.